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Cbx3 Inhibits Vascular Smooth Muscle Cell Proliferation, Migration and Neointima Formation

Running Title: Cbx3 in VSMC functions and neointima formation

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Abstract

Objective: To investigate the role of chromobox protein homolog 3 (Cbx3) in vascular smooth muscle cell (VSMC) proliferation, migration and neointima formation following vascular injury.

Methods and Results: Overexpression of Cbx3 led to a significant increase in VSMC contractile gene expression and VSMC apoptosis, as well as a dramatic decrease in collagen gene expression, VSMC proliferation and migration. Meanwhile the opposite was observed following inhibition of endogenous Cbx3. Luciferase activity assays revealed that Notch signalling, but not β–catenin nor NF-κB signalling, is regulated by Cbx3 in VSMCs, and among the four Notch receptors, Notch3 is selectively down-regulated by Cbx3 through a transcriptional repression mechanism. Notch3 gene activation recapitulates the effects of Cbx3 knockdown on VSMC proliferation and migration. Consequently, the inhibitory effects of Cbx3 over-expression on VSMC proliferation and migration were reversed by Notch3 gene re-activation. In a model of vascular damage by carotid wire injury, we observed that Cbx3 expression was dramatically down-regulated in injured arteries. Local ectopic overexpression of Cbx3 in injured arteries significantly inhibited Notch3 expression, thereby reducing VSMCs proliferation and causing an overall decrease in neointima formation. Additionally, injury-induced neointimal SMC hyperplasia was significantly reduced by aortic inhibition of Notch3. Importantly, a decreased expression level of Cbx3, but an increased expression level of Notch3 was observed in human femoral arteries with atherosclerotic lesions.

Conclusions: Cbx3 modulates VSMC contractile and collagen gene expression, as well as VSMC proliferation, migration, and apoptosis via a Notch3 pathway, and plays an important role in controlling injury-induced neointima formation.

Introduction

Vascular disease is the primary cause of myocardial infarction, stroke, and ischemia. Arterial remodeling characterized by alterations in the structure and function of the vascular wall in response to disease, injury, or aging, is the main underlying cause for various vascular diseases. The pathological process of arterial remodeling has been extensively characterized and involves a series of pro-inflammatory processes, endothelial dysfunction, vascular smooth muscle cell (VSMC) proliferation and migration, arterial calcification, as well as extracellular matrix remodeling[1-4]. Evidence accumulated from animal studies and histological observations in human has provided clear confirmation that VSMC proliferation and migration are two major local biologic processes influencing the process of post-angioplasty restenosis[1-4]. Therefore, exploring the underlying molecular mechanisms required for VSMC proliferation and migration is key to developing novel therapeutic strategies aimed at treating vascular disease.

Studies have suggested that VSMC migration and proliferation is mediated by a fairly complicated regulatory network comprised of growth factors, adhesion molecules[5-7], proteases[8], intra/extra-cellular proteins as well as non-coding RNAs[9, 10]. Moreover, it has been well established that VSMC migration and proliferation is closely governed by three key signal pathways, namely β-catenin[7, 11, 12], nuclear factor-κB (NF-κB)[13-15] and Notch signaling[16-18].

Chromobox protein homolog 3 (Cbx3) or heterochromatin protein 1γ (HP1γ), was primarily reported to regulate gene transcriptional silencing in heterochromatin-like complexes by promoting the conversion of local chromatin to a 'heterochromatin-like' repressive state through recognizing and binding to tri-methylated H3K9[19, 20]. However, later studies indicate that Cbx3 is a multifunctional molecule, and plays an important and diverse role in gene transcription[21], epigenetic regulation[22], DNA damage response pathway[23], embryonic development[24], human telomeres[25] and various cellular functions such as cellular differentiation[24, 26], reprogramming[27] and senescence[28]. Importantly, a critical role for Cbx3 in VSMC differentiation from stem cells and vascular development was demonstrated in our previous study[29] which showed that Cbx3 promoted stem cell differentiation towards VSMC lineage. Disruption of Cbx3 in neural crest cells resulted in the death of chick embryos due to severe developmental defects of the branchial arch arteries. Our study also revealed that Cbx3 mediates expression of SMC specific genes by regulating serum response factor (SRF) recruitment to the SMC gene promoters. Moreover, we showed that the interaction between Cbx3, Dia-1 and SRF plays a crucial role in Cbx3-mediated VSMC-specific gene expression. However, the potential significance of Cbx3 in mature VSMC functions and neointimal VSMC hyperplasia remains unclear. In the present study, we sought to investigate the effect of Cbx3 on VSMC proliferation and migration, and delineate any potential molecular mechanisms involved, specifically in the context of neointima formation following vascular injury.

Methods

Full Materials and Methods are available in the online only Data Supplement.

Animal experiments, anaesthesia and euthanasia

All animal experiments were conducted according to the Animals (Scientific Procedures) Act of 1986 (United Kingdom). All the animal procedures were approved by Queen Mary University of London ethics review board (PPL number: 70/7216), and conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the NIH guidelines (Guide for the care and use of laboratory animals). For mouse carotid artery denudation injury and gene delivery, anaesthesia was induced using 100% O_2/4% isoflurane, and was maintained throughout the injection by the administration of 100% O_2/2% isoflurane. At the end of protocol, all mice were euthanized by placing them under deep anaesthesia with 100% O_2/5% isoflurane, followed by decapitation.

Mouse carotid artery denudation injury and lentiviral particle infusion

C57BL/6 mice were anesthetized and the surgical procedure was similar to that described previously[8, 30-32]. Briefly, the left common carotid artery was dissected and injured by passing a curved flexible wire (0.38-mm diameter, Reference Part Number: C-SF-15-20, Cook Medical European Shared Services, Ireland) three to five times. After the vascular injury, the injured carotid arteries were randomly received lenti-GFP or lenti-Cbx3 treatments. The procedures for local gene delivery were similar to that described in the previous studies[33-39] with some modifications.

Morphometric analysis and quantification of lesion formation

The carotid arteries (~5.0mm from injury site) were harvested at 4 weeks post- operation. The specimens were fixed in 4% formaldehyde for H&E staining. Sections (8μm) were collected at 200μm intervals (10 sections per segment/interval), mounted on slides, and numbered. Five digitised sections with same identification number from five segments/intervals (~0.5mm, 1.5mm, 2.5mm, 3.5mm and 4.5mm from injury site) of each animal (e.g. I-1/2, III-1/2, V-1/2 represent the 1st and 2nd section of the 1st , 3rd and 5th segment/interval, respectively) were stained with H&E for morphometric analysis. The procedure used for lesion quantification was similar to that described in our previous studies[8, 9, 31, 32].

VSMC culture and treatments

Primary murine VSMCs were isolated from mouse aorta, and routinely maintained in DMEM supplemented with 10% FBS as described in our previous studies[8, 9]. A minimum of three different batches of VSMCs between passages 5 to 8 were used in the experiments described in the current study. VSMCs were treated with various athenogenic stimuli as described in our previous study[9].

VSMC proliferation/migration assays

VSMC proliferation was evaluated using 5-Bromo-2'-deoxy-uridine (BrdU) Labeling and Detection Kit II (Roche) according to the manufacturer's instructions and manual cell counting. VSMC migration was evaluated using scratch wound healing assays and trans-well migration assays, respectively, as described in our previous study[40].

Plasmid electroporation into primary VSMCs

Respective control or gene over-expression plasmids as indicated in the figure legend were transfected into primary VSMCs using Neon® Transfection System (MPK500S, Thermo

Fisher Scientific) with either 10µl or 100 µl Electroporation Pipette Tips, according to the manufacturer's instructions. Using this electroporation system, a very high transfection efficiency (>85%) is normally achieved with primary VSMCs in our Laboratory.

Human health and diseased arteries collection

Human diseased arteries and their neighbouring health tissues were collected and described in our recent study[41]. Briefly, human femoral arterial specimens were obtained from patients with peripheral arterial diseases undergoing leg amputation at the First Affiliated Hospital of Zhejiang University (China) between July 2014 and June 2017. All patients gave their written, informed consent. All procedures had local ethical approval (2014/294). All studies were approved by the Research Ethics Committees of the First Affiliated Hospital of Zhejiang University and all experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Statistical analysis. Each experiment was performed at least in biological triplicate, and all values are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using Graphpad Prism5. Shapiro-Wilk Normality Test was used for checking the normality of the data. Two tailed unpaired student's t-test was used for comparisons between 2 groups, or two-way analysis of variance (ANOVA) with a Bonferroni post-hoc test was applied when more than two groups were compared if the data displayed a normal distribution. Conversely, non-parametric Mann-Whitney U Test or Kruskal–Wallis one-way ANOVA with a post hoc test of Dunn's test was applied for comparing two groups and three or more groups, respectively, if the data did not display normal distribution or if the number of observations from each group was smaller than 5 ($n<5$). $P<0.05$ was considered statistically significant.

Results

Impact of Cbx3 on VSMC contractile and collagen gene expression

We previously reported that Cbx3 promotes SMC differentiation from stem cells by controlling VSMC contractile gene expression[29]. To explore the potential role of Cbx3 in mature VSMC differentiation and dedifferentiation, Cbx3 over-expression and knockdown experiments were conducted in VSMCs. Data from quantitative real-time reverse transcription-PCR (qRT-PCR) analyses showed that a panel of VSMC contractile genes (SMαA, S22αA, h1-calponin, and SM-myh11) were significantly up-regulated by Cbx3 over-expression. Conversely, Cbx3 over-expression led to a decreased expression level of two collagen genes (col1a1, col4a1) (**Figure S1A**). As expected, Cbx3 knockdown exerted the opposite effect for gene expression (**Figure S1B**). The expression levels of the other four collagen genes examined (col2a1, col3a1, col5a1, and col6a1) were not affected by Cbx3 in VSMCs (**Figure S1A and S1B**).

Effect of Cbx3 on ex vivo VSMC proliferation, migration, and apoptosis

To study if Cbx3 has any effect on VSMC proliferation, we compared the proliferation rate of VSMCs transfected with a control plasmid and a Cbx3 over-expressing plasmid. qRT-PCR and Western blotting analyses confirmed that Cbx3 was successfully over-expressed in VSMCs at both mRNA and protein levels (**Figure 1A and 1B**). BrdU incorporation assays (**Figure 1C**) and cell counting (**Figure S2A**) revealed that Cbx3 over-expression in VSMCs results in a significantly lower rate of proliferation than VSMCs transfected with the control plasmid in response to serum and PDGF-BB stimulation. To substantiate these findings, we measured the effect of Cbx3 knockdown on VSMC growth by comparing cell growth rate of the VSMCs infected with Cbx3 shRNA lentivirus to VSMCs infected with a non-target shRNA lentivirus. As expected, we observed that over 70% of endogenous Cbx3 was inhibited by Cbx3 shRNA in VSMCs at both the mRNA (**Figure 1D**) and protein (**Figure 1E**) level. Consequently, we found that Cbx3 inhibition significantly increased VSMC proliferation as assessed using BrdU incorporation assays (**Figure 1F**) and cell counting (**Figure S2B**). All the above data provides conclusive evidence that Cbx3 has an inhibitory effect on VSMC proliferation.

To investigate whether Cbx3 has an effect on VSMC migration, we performed *in vitro* scratch wound healing assays and Transwell migration assays on cultured VSMCs. The assays showed that compared to controls, Cbx3 overexpression limits the ability of VSMCs to migrate, while Cbx3 knockdown has the opposite effect (**Figure 1G-H, Figure S2C-D, Figure S3, and Figure S4**).

VSMC apoptosis is another major contributor to vascular disease, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was conducted in VSMCs to determine if Cbx3 plays a role in VSMC apoptosis. We observed that VSMC apoptosis was significantly increased by Cbx3 over-expression, but decreased by Cbx3 inhibition (**Figure S5**), proving that Cbx3 promotes VSMC apoptosis.

Effect of Cbx3 on Notch, β-catenin and NF-κB signaling pathway in VSMCs

As mentioned earlier, Notch, β-catenin and NF-κB signaling are three major pathways involved in VSMC pathology and human disease. As such, we wondered whether one or more of these signaling pathways were responsible for the inhibitory effects of Cbx3 on VSMC proliferation and migration. To this end, VSMCs were co-transfected with the Cbx3 over-expression plasmid as well as a reporter vector containing either four copies of a CSL binding site (pGL3-4xCSL for measuring Notch signaling, a gift from Raphael Kopan[42] (Addgene plasmid # 41726)), seven copies of a TCF/LEF binding site (M50 Super 8x TOPFlash for measuring β-catenin signaling, a gift from Randall Moon[43] (Addgene plasmid # 12456)) or five copies of an NF-κB response element (NF-κB-RE) (pGL4-NF-κB or

pGL4.32[luc2P/NF-κB-RE/Hygro] for measuring NF-κB signaling, Promega, E8491). Luciferase activity assays showed that the reporter activity of pGL3-4xCSL, but not that of TopFlash and pGL4-NF-κB, was significantly inhibited by Cbx3 over-expression in VSMCs (**Figure S6**), revealing an inhibitory role for Cbx3 in Notch signaling.

Cbx3 down-regulates Notch3 through a transcriptional repressing mechanism

Since our data shows that Cbx3 inhibits Notch signaling in VSMCs and the Notch signaling network comprises four Notch receptors (Notch 1-4), we decided to investigate which Notch receptor(s) were affected by Cbx3 in VSMCs. We examined the gene expression levels of Notch 1 to 4 in VSMCs transfected with control and Cbx3 over-expressing plasmids, and found that only Notch3 gene expression was significantly down-regulated by Cbx3 over-expression (**Figure 2A and Figure S7**). Inhibition of Notch3 expression by Cbx3 over-expression was also confirmed at the protein level (**Figure 2B**). Conversely, we found that knockdown of endogenous Cbx3 caused a significant increase in Notch3 expression at both the mRNA and protein level (**Figure 2C-D**), confirming an inhibitory effect for Cbx3 on Notch3 gene regulation. Luciferase activity assays showed that Notch3 promoter activity was significantly inhibited by Cbx3 expression (**Figure 2E**), indicating that Cbx3 inhibits Notch3 gene expression through a transcriptional mechanism. Data from CHIP assays revealed that Cbx3 can directly bind to Notch3 gene promoter DNA, an effect that was enhanced by Cbx3 over-expression in VSMCs (**Figure 2F**). Collectively this data demonstrates that Cbx3 inhibits Notch3 gene expression through a transcriptional repression mechanism.

Notch3 gene activation recapitulates the effects of Cbx3 knockdown on VSMC proliferation and migration

To investigate the functional impacts of Notch3 on VSMC proliferation and migration, we compared the cell proliferation rate and migration capability between control and Notch3 over-expressing VSMCs. For this purpose, serum starved VSMCs were transfected with a control vector or a Notch3 functional domain (namely Notch3 Intracellular Domain (NICD3)) over-expression plasmid (NICD3 or pCMV-3xFlag-NICD3, a gift from Raphael Kopan[44], Addgene, plasmid 20185), and subjected to the following assays. Gene expression data obtained by qRT-PCR revealed that the expression level of Notch3, but not Cbx3, was significantly up-regulated by the NICD3 over-expression plasmid (**Figure 3A**). Proliferation assays showed that Notch3 over-expression promoted VSMC growth assessed by cell counting (**Figure 3B**) and BrdU incorporation analysis (**Figure 3C**). Data from transwell migration assays also revealed that Notch3 over-expressing VSMCs had a significantly higher migratory capacity in response to both serum and PDGF-BB stimulation (**Figure 3D**). Hence, the above data reveals that Notch3 has a positive effect on VSMC proliferation and migration.

Notch3 gene re-activation abolishes the inhibitory effects of Cbx3 on VSMC proliferation and migration

To investigate whether modulation of the Notch3 gene is responsible for Cbx3-mediated inhibition of VSMC proliferation and migration, Cbx3 and Notch3 were co-overexpressed in VSMCs, using control and/or over-expression plasmids, as indicated in the figures, followed by VSMC proliferation and migration assays. qRT-PCR analysis revealed that both Cbx3 and Notch3 were successfully up-regulated (**Figure 4A**). However, Notch3 expression was significantly down-regulated by Cbx3 over-expression, while the expression levels of Cbx3 were not altered by Notch3 over-expression, confirming that Notch3 is the downstream target gene of Cbx3 in VSMC pathology. Importantly, BrdU incorporation assays showed that while VSMCs transfected with Cbx3 (2nd bars) or Notch3 (3rd bars) over-expressing plasmid

alone exhibited a significant lower or higher cell proliferation rate, respectively, re-activation of Notch3 abolished the inhibitory effects of Cbx3 over-expression on VSMC proliferation (4th bars) (**Figure 4B**). A similar phenomenon was observed in VSMC transwell migration assays (**Figure 4C**). Thus the above data reveals an important relationship between Cbx3 and Notch3 in the context of VSMC migration and proliferation.

Impact of locally enforced expression of Cbx3 and inhibition of Notch3 on VSMC proliferation and neointimal SMC hyperplasia after vessel injury

To investigate if Cbx3 plays a role in vessel injury-induced neointima formation, a well-established arterial remodelling model[8, 30] was carried out in mice. In this model, wire-induced vessel injury results in rampant VSMC migration and proliferation, peaking between day 7 and day 10 and resulting in the formation of a neointima which progresses until 28 days following injury [8, 45]. Consistent with our *in vitro* data, we observed that the expression level of Cbx3 was decreased as early as Day 3 post-injury, and was significantly down-regulated between week 1 and week 2 (**Figure 5A**), suggestive of a protective role for Cbx3 that is naturally absent during neointima formation and progression after injury. To facilitate our *in vivo* investigations, we generated Lenti-GFP (control) and Lenti-Cbx3 lentiviral particles, and directly infused them into the lumen of the injured carotid arteries immediately after vessel injury to induce local VSMC infection. Double immunofluorescence staining using antibodies against GFP and SMA (SMC marker), CD68 (macrophage marker), or CD31 (endothelial cell marker) confirmed a SMC-specific infection since GFP-positive cells within injured arterial wall co-expressed SMA (**Figure S8A**), but not CD68 (**Figure S8B**) or CD31 (**Figure S8C**). We found that direct infusion of Lenti-Cbx3 into the injured arteries significantly increased the expression levels of Cbx3 (**Figure 5B-C**) in the injured arteries compared to the injured vessels treated with the control lentivirus. Compared with the normal uninjured arteries, the expression levels of Notch3, PCNA (cell proliferation marker), and collagen (col1a1 and col4a1) were significantly increased in the injured vessels infected with Lenti-GFP, but this effect was reversed by Lenti-Cbx3 (**Figure 5B**). This provided confirmation of the *in vivo* inhibitory effect of Cbx3 on Notch3 and collagen gene expression in the injured arteries, and demonstrated that locally enforced expression of Cbx3 in injured vessels decreases VSMC proliferation. Importantly, we observed an approximate 50% decrease in neointima formation in the injured vessels infected with Lenti-Cbx3, compared with the injured arteries infected with Lenti-GFP (**Figure 5D and 5E**). Specifically, a significant decrease in neointimal hyperplasia (neointimal area, 11,000±1,250 μm^2 versus 20,500±2,025 μm^2) and neointima/media ratio (1.14±0.19 versus 2.09±0.29) was observed in the injured vessels infected with Lenti-Cbx3 at 28 days post-injury compared with control injured vessels (**Figure 5E**).

To better understand the inhibitory effect of Cbx3 on VSMC migration and proliferation within the context of injury-induced neointima formation, we analyzed the impact of locally enforced expression of Cbx3 on neointima formation after injury at an early time point (day 10 post-injury). At this time-point VSMC migration is the more predominant event, more so than VSMC proliferation when considering their relative contributions to injury-induced neointima formation. Data shown in **Figure 5F and 5G** revealed that local infusion of Lenti-Cbx3 led to a trend of decreased neointima SMC hyperplasia after injury, although this did not reach significance, indicating that the inhibitory effect for Cbx3 on VSMC migration has an effect on the response to vascular injury at 4 weeks post-injury, but such an effect cannot be detected at an early stage (10 days post-injury).

Our data demonstrated that Notch3 was up-regulated in response to vascular injury, and this was reversed by Cbx3 over-expression in injured arteries (**Figure 5B and 5C**). To further confirm the functional importance of Notch3 activation in vascular injury-induced neointima

formation, control or Cbx3 shRNA lentiviral particles were directly infused into the lumen of the injured carotid arteries immediately after vessel injury. We observed that local infusion of Cbx3 shRNA lentiviral particle resulted in a significant decrease in Notch3 gene expression in injured arteries, with no obvious change for Cbx3 gene expression (**Figure 5F**). Combined with the above findings, showing that Notch3 gene expression is modulated by Cbx3 over-expression in injured arteries (**Figure 5B and 5C**), our data further confirms a regulatory role for Cbx3 on Notch3 gene expression in neointima formation after injury. As expected, PCNA gene expression was decreased by local inhibition of Notch3 (**Figure 5H**). Consequently, we found local inhibition of Cbx3 led to an approximately 55% decrease in neointima formation (**Figure 5I and 5J**).

Cbx3 and Notch3 expression in diseased human arteries and its neighbouring segments

To further explore the functional relevance of the Cbx3-Notch3 regulatory axis in a clinical setting, Cbx3 and Nocth3 gene expression and their relationship was examined in eight pairs of femoral arterial specimens (arterial fragments with atherosclerotic lesions and their respective neighbouring segments with less apparent disease) from patients with peripheral arterial diseases undergoing leg amputations collected in our recent study[41]. Double immunohistochemistry staining assay showed that VSMCs in human arteries express both Cbx3 and Notch3 (**Figure S9**). Importantly, we observed a decreased expression level of the Cbx3 gene, with an increased gene expression level of Notch3 in the diseased femoral arteries, compared with their neighbouring segments with less apparent disease (**Figure 6A**). Meanwhile, we noted a significant inverse relationship and a trend pointing towards an inverse association between Cbx3 and Notch3 in the diseased femoral arterial specimens as well as its neighbouring segments with less apparent disease, respectively (**Figure 6B**).

Discussion

VSMC proliferation, migration, and apoptosis are the key biological processes occurring locally after vascular injury, which influence the rate of post-angioplasty restenosis, as well as vascular disease formation and progression[1-4]. As such, signaling pathways controlling these VSMC functions represent potentially novel therapeutic drug target for the treatment of vascular disease. Our findings have provided new insights into the intracellular signaling pathways governing VSMC proliferation, migration, apoptosis, and neointima formation. Specifically, we found a decreased expression of Cbx3 in injured arteries, and have shown that Cbx3 has an inhibitory effect on VSMC pathology and adverse arterial remodeling in response to injury. Mechanistically, we have provided compelling evidence to show that Cbx3 exerts its inhibitory effect on VSMC proliferation, migration, apoptosis, and injury-induced neointimal SMC hyperplasia through modulation of Notch3 gene expression in VSMCs and injured arteries. We have confirmed the clinical relevance of Cbx3-Notch3 regulatory axis in human femoral arteries with or without atherosclerotic lesions. The finding presented in the current study will significantly enhance our knowledge of the etiology of vascular disease, and potentially pave a way to develop a new therapeutic agent for preventing vascular disease.

Accumulating evidence indicates that Cbx3/HP1γ is an important regulator of several biological processes, including pluripotent stem cell fate decision[24, 46], neural differentiation[26]/maturation[47], embryonic development, primordial germ cell formation[48], spermatogenesis[49], adipogenesis[50], cell mitosis[51] or meiosis[52], DNA damage response pathway[23, 53, 54], cellular senescence[28], and reprogramming[27]. Cbx3 dysregulation has also been implicated in various human diseases such as cancer[55-58], dyskeratosis congenita[25] and facioscapulohumeral dystrophy[59]. In relation to VSMCs, our previous work[29] has identified Cbx3 as a critical regulator of VSMC differentiation and embryonic vascular system development both *in vitro* and *in vivo*. Moving forwards, we have now provided new evidence to show that Cbx3 also plays an important role in VSMC pathology. Data from Cbx3 over-expression and knockdown assays show that VSMC proliferation and migration, as well as VSMC apoptosis and collagen synthesis, which are the critical cellular events in vascular neointimal lesion formation, are controlled by Cbx3. Moreover, by manipulating the expression level of Cbx3 in the injured vessels, we found locally enforced expression of Cbx3 in the injured carotid arteries significantly reduces VSMC proliferation, and inhibits neointima formation at 28 days post-injury (**Figure 5D and 5E**), suggesting that Cbx3 is a potential therapeutic target in post-angioplasty restenosis. Surprisingly, we found that at an early time point (10 days post-injury), when VSMC migration is the more predominant event than VSMC proliferation, the inhibitory effect of locally enforced expression of Cbx3 on neointima formation after injury is much less apparent than that at later stage (28 days post-injury) (**Figure 5F and 5G**). Following vascular injury, VSMCs from the arterial tunica media are activated and migrate to the intima, where they massively proliferate, and eventually lead to neointimal thickening and restenosis. Therefore, our data indicates that the combined inhibitory effects of Cbx3 on VSMC migration and proliferation contribute to the decreased neointima SMC hyperplasia after injury at later stage.

One of the novel findings in our current study is that Notch3 has been identified as the functional downstream target of Cbx3 in VSMC pathology. We have shown that Notch3 is selectively regulated by Cbx3 in VSMCs through a transcriptional repression mechanism, which is consistent with the well-established function of Cbx3 as a transcriptional repressor in gene regulation. It was initially reported that Cbx3/HP1γ, a nonhistone chromosomal protein, plays a dose-dependent role in gene silencing through binding to methylated H3K9[19, 20]. Such an interaction is further facilitated by nucleosome compaction[60]. Apart from recognizing and binding to methylated H3K9, Cbx3 plays a decisive but important role in

gene regulation through interaction with distinct binding partners. Multiple mechanisms have been suggested as the underlying molecular mechanisms through which Cbx3 regulates gene expression. For instance, it has been reported that Cbx3 regulates its target gene expression through modulation of RNA processing[61] or alternative splicing[62, 63]. Cbx3 has also been identified as an epigenetic regulator of human embryonic ϵ-globin gene expression[64]. Conversely, a transcriptional activation role has also been attributed to Cbx3. Studies have shown that acute HSP70 gene transcription is regulated by the physical interactions between the histone variant H3.3 and HP1γ at HSP70 gene promoter[65]. HP1γ also participates in NF-κB-dependent gene regulation in activated macrophages through interaction with IκB kinase-α[66]. Our own data also demonstrates that Cbx3 regulates VSMC specific gene expression through a transcriptional activation mechanism during VSMC differentiation from stem cells[29]. In our current study, we have shown that Notch3 is a transcriptional target of Cbx3 in the context of VSMCs. Both Notch3 RNA and protein expression levels were significantly down-regulated by Cbx3 over-expression, but dramatically up-regulated when the endogenous Cbx3 expression was blocked in VSMCs. Cbx3 also significantly inhibits Notch3 promoter activity, inferring that Cbx3 regulates Notch3 at transcriptional level. CHIP assays revealed that Cbx3 directly binds to the promoter region of the Notch3 gene, which is consistent with previous findings. Choi[21], et al reported that HP1γ controls the suppression and recovery of breast cancer type 1 susceptibility protein (BRCA1)-mediated gene transcription through direct binding to the BRCA1 target gene promoters.

In our current study, we showed that Notch3 promotes VSMC proliferation and migration, and repression of Notch3 is required for Cbx3-mediated regulation/inhibition of VSMC proliferation and migration. We have also provided evidence to show that Notch3 is negatively regulated by Cbx3 during arterial remodeling in response to injury, inferring a role for Cbx3-Notch3 axis in vascular diseases, which is consistent with previously reported roles of Notch3 in VSMC pathology, development and cardiovascular diseases. Notch3 is predominantly expressed in adult arterial SMCs, and mutations within its gene causes cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoecephelopathy (CADASIL), an inherited early stroke syndrome leading to dementia due to systemic vascular degeneration[67]. Accumulated evidence reveals that Notch3 plays a critical role in controlling VSMC behaviour and phenotype such as survival[68], apoptosis[69], proliferation[70], migration[71], trans-differentiation[72] or osteogenic differentiation[73], and is a key determinant of vascular development[74-76], pericyte coverage[77] or mural cell investment[78] during angiogenesis/arteriogenesis, and maintaining blood vessel integrity[79, 80]. Notch3 has also been implicated in various cardiovascular diseases such as CADASIL[81, 82], heart failure[83], cardiac fibrosis after myocardial infarction[84], arterial injury/remodeling[85] and pulmonary arterial hypertension[86]. Despite the huge scope of Notch3 involvement in many cardiovascular diseases, its significance and functional relevance in injury-induced neointima SMC hyperplasia was refuted by a genetic study, in which the authors reported that genetic deletion of Notch3 does not affect neointima formation in response to vascular injury[87]. Conversely, we have now provided supporting evidence to suggest that Notch3 activation in response to injury does contribute to neointima SMC hyperplasia since we observed a significant decrease in neointima size and a reduced neointima/media ratio in injured arteries treated with Notch3 shRNA lentivirus (**Figure 5H-J**). This disparity may be attributable to the fact that some compensatory mechanisms are activated in mice following global Notch3 gene deletion.

Taken together, we have documented an important role for Cbx3 in VSMC pathology and injury-induced adverse arterial remodeling, and identified Notch3 as the functional downstream target gene that is responsible for Cbx3-mediated VSMC function and neointima formation. However, it is worth mentioning that endothelial dysfunction, poor endothelial

coverage, as well as monocyte/macrophage and lymphocyte infiltration into the injured arterial wall are also key contributors to vascular disease. Therefore, the potential effects of Cbx3 on endothelial coverage (re-endothelialization), as well as monocyte/macrophages and lymphocyte function, and their contribution to vascular remodeling warrant further investigation, but unfortunately fell beyond the remit of our current study. Nevertheless, the findings presented in our study significantly enhance our knowledge of signaling pathways that govern VSMC proliferation and migration both *in vitro* and *in vivo*, and provide us with a novel therapeutic target within the Cbx3/Notch3 signaling pathway that has the potential for treating vascular disease.

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None

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Figure Legends

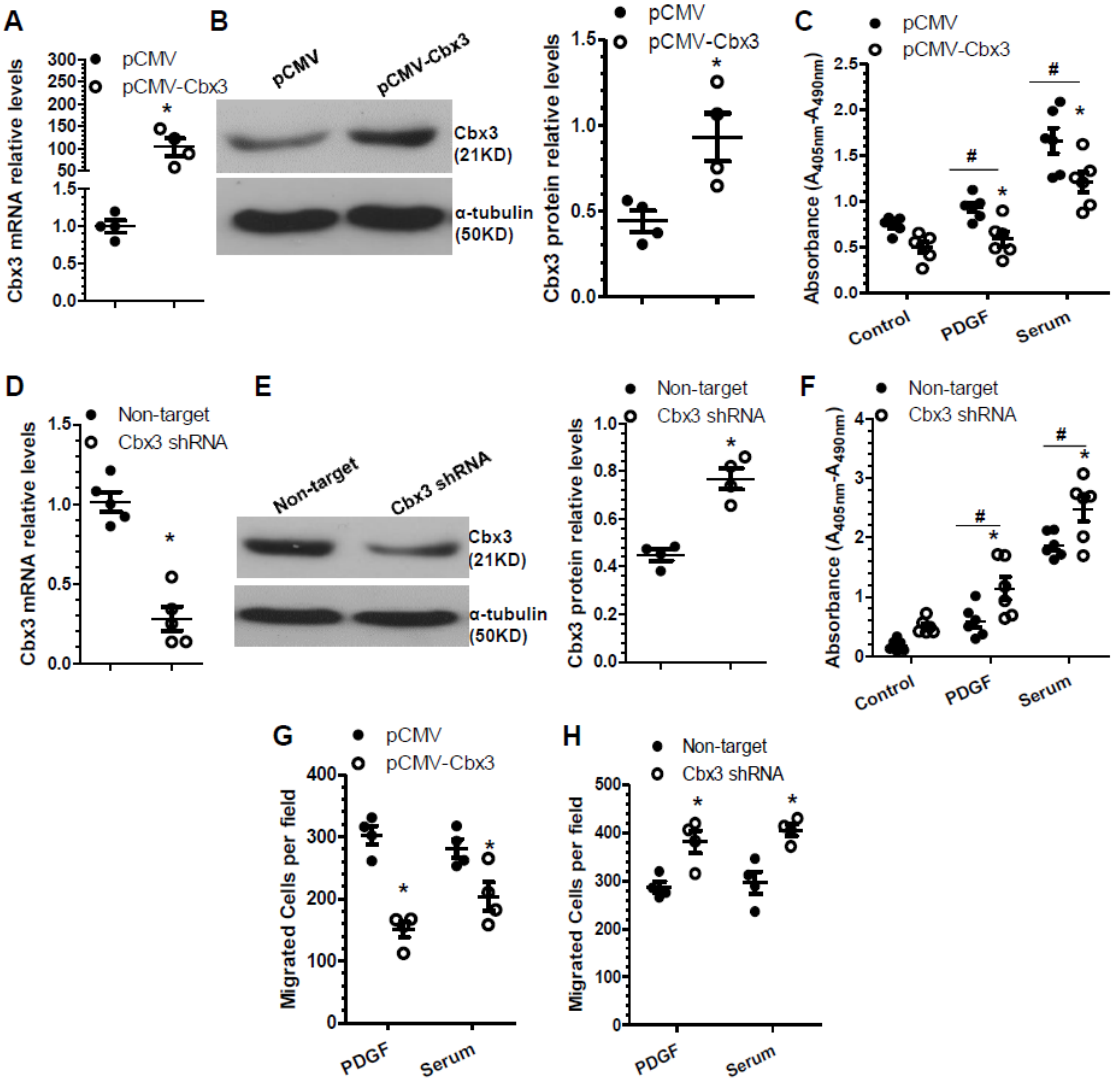

Figure 1. VSMC proliferation and migration are controlled by Cbx3.
(A) qRT-PCR confirmed Cbx3 over-expression in VSMCs. (**B**) Left Panel: Protein expression of Cbx3 in VSMCs transfected with control or Cbx3 over-expression plasmid. The expression of α-tubulin was used as an internal control. Right Panel: The quantitative results of four independent experiments. (C) Cbx3 over-expression inhibited VSMC proliferation. Cultured VSMCs transfected with control (pCMV) or Cbx3 over-expression (pCMV-Cbx3) plasmids were subjected to serum starvation for 24~48 hours. After then, cells were treated with 20% serum and 10ng/ml PDGF-BB for further 48 hours, followed by BrdU assays. (D) qRT-PCR analyses confirmed that endogenous Cbx3 was inhibited in VSMCs by Cbx3 shRNA. (E) Left Panel: Protein expression of Cbx3 in VSMCs infected with non-target or Cbx3 shRNA lentivirus. The expression of α-tubulin was used as an internal control. Right Panel: The quantitative results of four independent experiments. (F) Knockdown of Cbx3 promoted VSMC proliferation. VSMCs were infected with control (non-target shRNA) or Cbx3 shRNA lentivirus, and subjected to similar treatments and assays as described in (C). (G-H) VSMC migration was inhibited by Cbx3 over-expression (G), but increased by Cbx3 knockdown (H). Cultured VSMCs transfected with pCMV-Cbx3 or infected with Cbx3 shRNA lentivirus, or respective control vectors (pCMV or non-target shRNA) were subjected to serum starvation for 48 hours. After which, cells were subjected to trans-well migration analyses in the presence of PDGF-BB or serum stimulation as indicated. Note: in the trans-

well migration experiments, only a few cells migrated through the insert without any chemoattractant. The data presented here are mean±S.E.M. of four (n=4 in A, B, D, E, G and H; Mann-Whitney U Test) or six (n=6 in C and F; two-way ANOVA with a Bonferroni post-hoc test) independent experiments. *P<0.05 (versus pCMV or non-target shRNA), #P<0.05 (PDGF/serum versus control).

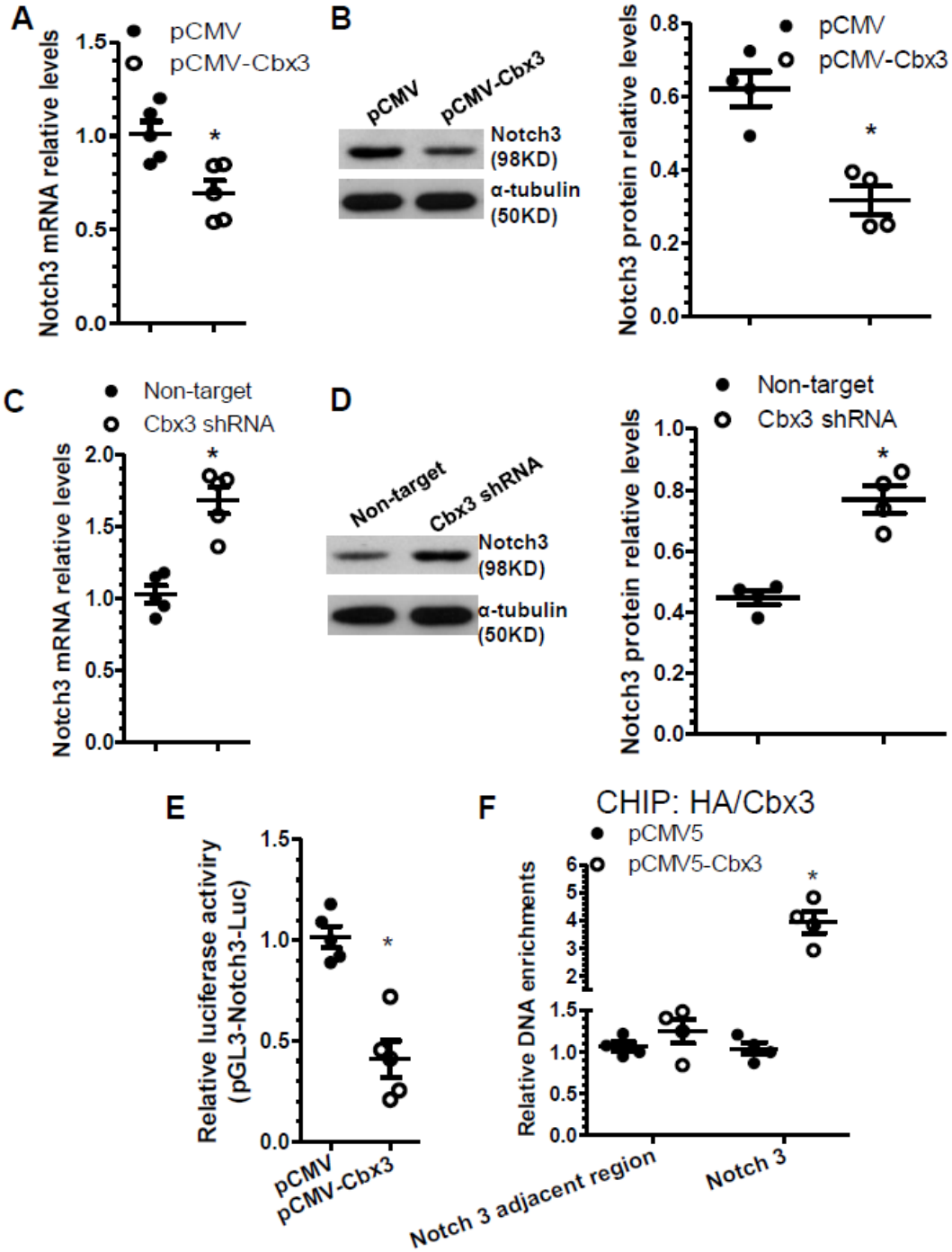

Figure 2. Cbx3 down-regulates Notch3 through a transcriptional repression mechanism. (A-D) Notch3 expression was down-regulated by Cbx3 over-expression (A and B), but up-regulated by Cbx3 knockdown (C and D). The data presented here are representatives or mean±S.E.M. of five (A and C, *P<0.05 (versus pCMV or non-target shRNA), t-test) or four (B and D, *P<0.05 (versus pCMV or non-target shRNA), Mann-Whitney U Test)

independent experiments. (E) Notch3 promoter activity is inhibited by Cbx3 over-expression. Notch3 promoter reporter vector (pGL3-Notch3-Luc) was co-transfected with control (pCMV) or Cbx3 over-expression (pCMV-Cbx3) plasmid into VSMCs. Transfected cells were subjected to serum starvation for 24 hours, followed by incubation with DMEM supplemented with 20% serum for another 24 hours. Cells were harvested and cell lysate was subjected to Dual-luciferase activity assay with a standard protocol. Data presented here are mean±S.E.M. of five independent experiments (n=5). *P<0.05 (versus pCMV, t-test). (F) Cbx3 directly binds to Notch3 gene promoter. CHIP assays were conducted in VSMCs transfected with pCMV or pCMV-HA-Cbx3 using antibody against HA (Cbx3). Data presented here are mean±S.E.M. of four independent experiments (n=4). *P<0.05 (versus pCMV, Mann-Whitney U Test).

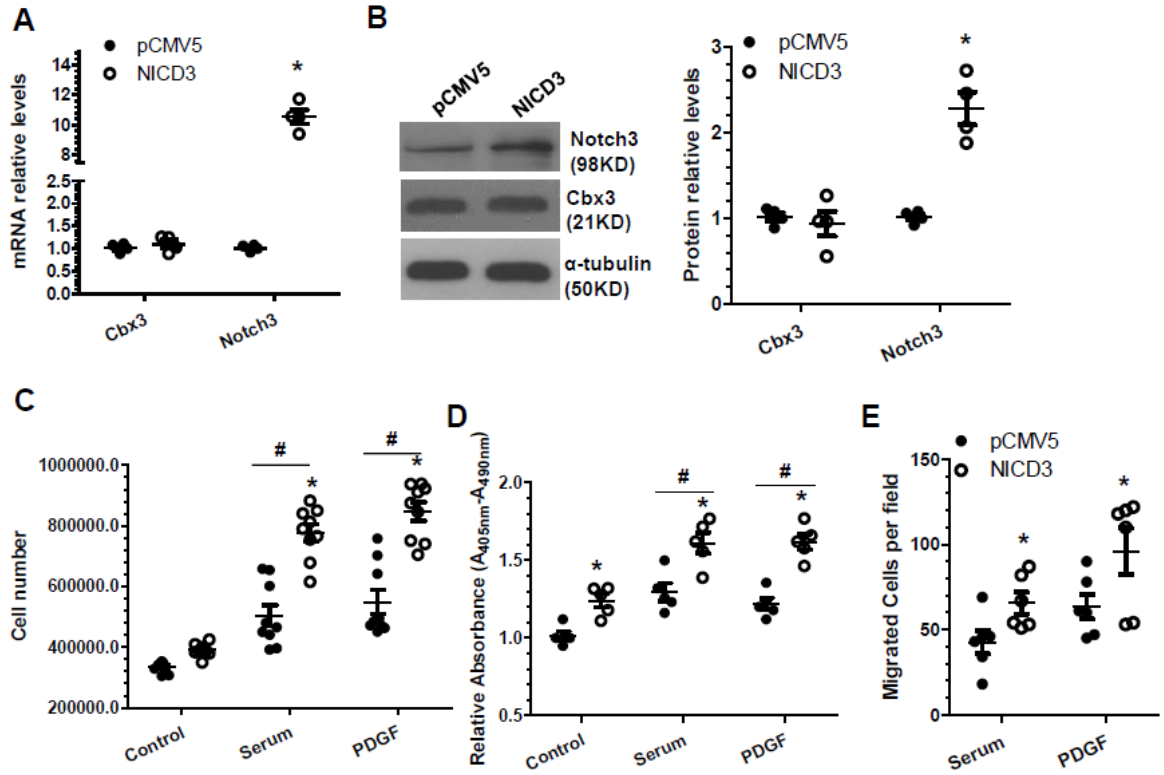

Figure 3. Notch3 activation recapitulates the effects of Cbx3 knockdown on VSMC proliferation and migration.

(A) qRT-PCR analyses confirmed Notch3 upregulation in VSMCs transfected with NICD3 over-expression plasmid (NICD3 or pCMV-3xFlag-NICD3). *P<0.05 (versus pCMV5, n=4, Mann-Whitney U Test). (B) Left Panel: Protein expression of Notch3 and Cbx3 in VSMCs transfected with control or NICD3 plasmid. The expression of α-tubulin was used as an internal control. Right Panel: The quantitative results of four independent experiments (n=4). *P<0.05 (versus pCMV5, Mann-Whitney U Test). (C and D) Notch3 promotes VSMC proliferation. Cultured VSMCs transfected with control (pCMV5) or Notch3 over-expression (NICD3) plasmids were subjected to serum starvation for 24~48 hours. After which, cells were treated with 20% serum and 10ng/ml PDGF-BB for further 48 hours, followed by cell counting (C) and BrdU assays (D) , respectively. (E) Notch3 increases VSMC migration. Cultured VSMCs transfected with control (pCMV5) or Notch3 over-expression (NICD3) plasmids were subjected to serum starvation for 24~48 hours, and followed by trans-well migration. Data presented here are mean±S.E.M. of nine (C) or five (D and E) independent experiments. *P<0.05 (versus pCMV5), #P<0.05 (PDGF/serum versus control), two-way

ANOVA with a Bonferroni post-hoc test and t-test were used for statistical analysis in C & D, and E, respectively.

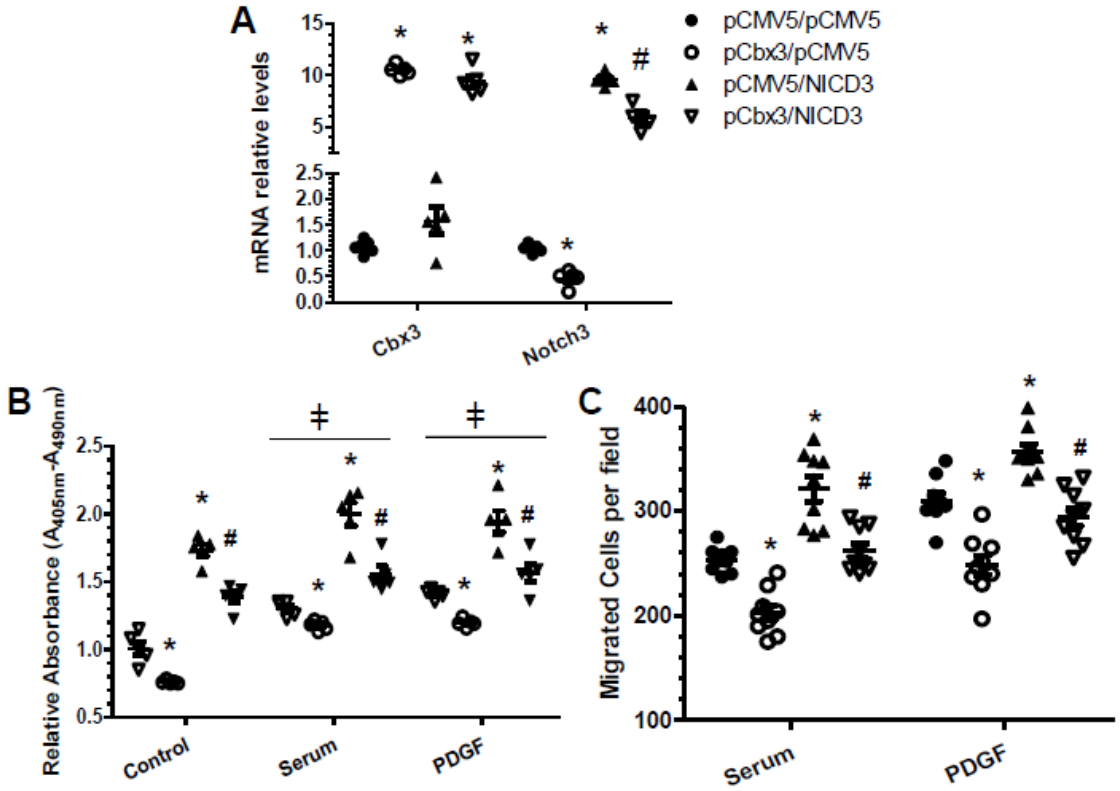

Figure 4. Notch3 gene re-activation abolishes the inhibitory effects of Cbx3 over-expression on VSMC proliferation and migration.
(A) Cbx3 and Notch3 gene expression levels in VSMCs transfected with Cbx3 or NICD3 over-expression plasmids. (B-C) The inhibitory effects of Cbx3 over-expression on VSMC growth and migration were abolished by re-activation of Notch3 gene. VSMCs were co-transfected with pCbx3 (Cbx3 over-expression), NICD3 (Notch3 over-expression), and/or respective control (pCMV5) as indicated. Transfected cells were serum-starved for 24 hours and subjected to BrdU incorporation (B) and trans-well migration (C) assays, respectively. The data presented here are mean±S.E.M. of five (n=5, A and B) or eight (n=8, C) independent experiments. *P<0.05 (Cbx3 or Notch3 over-expression versus control); #P<0.05 (NICD3 versus pCMV5 in the presence of pCbx3, 4th versus 2nd column); ǂ P<0.05 (PDGF/serum versus control); two-way ANOVA with a Bonferroni post-hoc test.

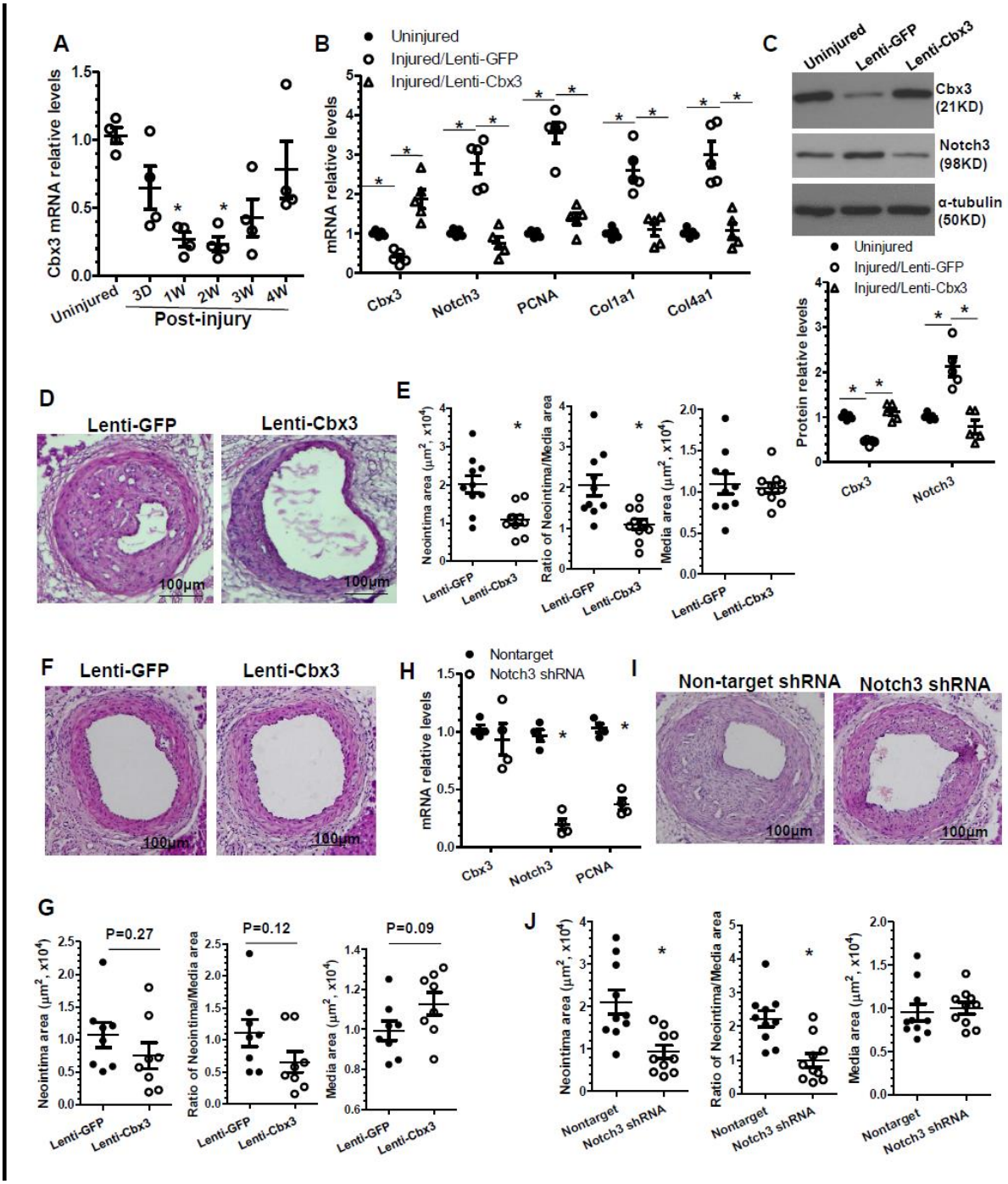

Figure 5. Neointima formation after injury was inhibited by enforced expression of Cbx3 or inhibition of Notch3 in injured arteries.

(A) Cbx3 gene expression is decreased in the injured vessels. Total RNAs were harvested from uninjured (used as sham surgery) and post-injury femoral arteries at the indicated times, and subjected to qRT-PCR analyses. Data presented here are mean±S.E.M. of four independent experiments (carotid arteries from 3~5 mice were pooled for each experiment, n=4 experiments). *P<0.05 (versus injured arteries; Kruskal–Wallis one-way ANOVA with a post hoc test of Dunn's test). (B) Gene expression levels in uninjured and injured arteries treated with control (Lenti-GFP) or Cbx3 (Lenti-Cbx3) lentivirus. After injury, 20μl of DMEM containing 1.0~2.0 x 10^6 lentiviral particles (Lenti-Cbx3 or Lenti-GFP) was directly infused into the lumen of the injured carotid arteries, followed by a 30-minute incubation for local VSMC infection. At seven days post-injury/treatment, total RNAs were harvested from

the injured segments of carotid arteries and subjected to qRT-PCR analyses. Data presented here are mean±S.E.M. of five independent experiments (carotid arteries from 3~5 mice were pooled for each experiment, n=5 experiments). *P<0.05 (two-way ANOVA with a Bonferroni post-hoc test). (C) Protein expression level in arteries subjected to same treatment as described in (B). Top panel: representative blots. Bottom panel: the quantitative results of five independent experiments (n=5). *P<0.05 (two-way ANOVA with a Bonferroni post-hoc test). (D-E) Local Cbx3 gene transfer inhibited neointima formation in wire-injured carotid arteries. At 28 days post-injury, injured segments of carotid arteries were harvested. Paraffin sections from both groups (n=10 mice per group) were prepared and subjected to H&E staining analyses. Representative images (D) and morphological characteristics (E) including neointimal area, neointimal/media (N/M) ratio, and media area of the injured arteries were presented here. *P<0.05 (Lenti-Cbx3 versus Lenti-GFP, t-test). (**F-G**) **Potential impact of local infusion of Lenti-Cbx3 on neointima formation at an early stage.** Mice were randomly received similar treatment as described in (D-E). At 10 days post-injury/treatment, injured segments of carotid arteries were harvested. Paraffin sections from both groups (n=8 mice per group) were prepared and subjected to similar analyses as shown in (D-E). t-test was applied in the statistical analysis. (**H**) Gene expression levels in injured arteries infected with control (non-target) or Cbx3 shRNA lentivirus as described in (B). Data presented here are mean±S.E.M. of five independent experiments (n=5). *P<0.05 (two-way ANOVA with a Bonferroni post-hoc test). (**I and J**) Local inhibition of Notch3 reduced neointima formation. At 28 days post-injury and shRNA lentivirus infection, injured segments of carotid arteries were harvested and subjected to H&E staining analyses. Representative images (G) and morphological characteristics (H) of the injured arteries from 10 mice (per group, n=10) were presented here. *P<0.05 (Notch3 shRNA versus non-target, t-test).

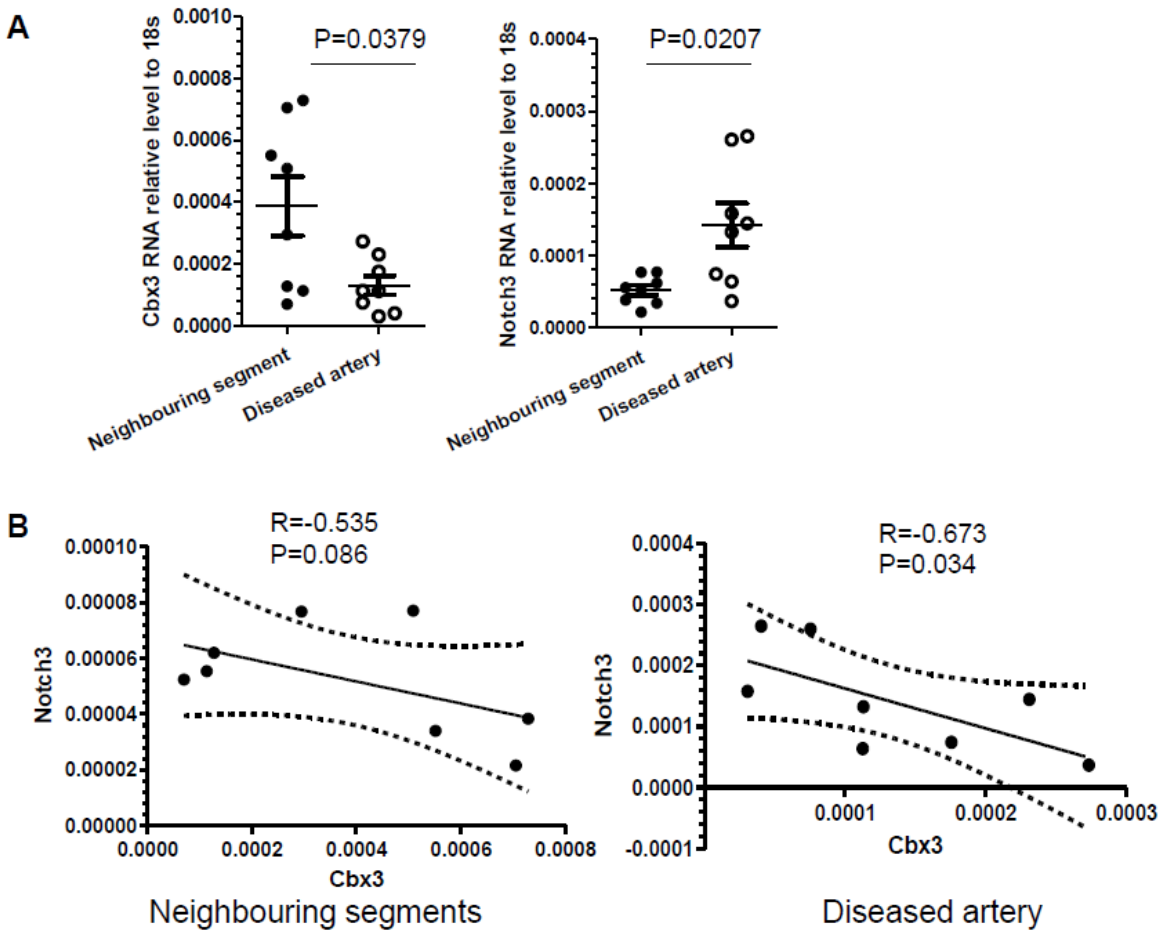

Figure 6. Cbx3 and Notch3 expression in diseased human arteries and its neighbouring segments with less apparent disease. Eight pairs of femoral arterial specimens (arterial fragments with atherosclerotic lesions (diseased artery) and their respective neighbouring segments with less apparent disease (neighbouring segments)) from patients with peripheral arterial diseases that underwent leg amputation were collected and subjected to qRT-PCR analyses. (A) Gene expression profiles in diseased arteries and its neighbouring segments with less apparent disease. Mann-Whitney U Test was applied for statistical analysis. (B) Pearson's correlation coefficient analyses of the gene expression levels of Cbx3 and Notch3 in human femoral arterial specimens. N=8 from each group.